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Attractive and repulsive interactions between female and male gametophytes in *Arabidopsis* pollen tube guidance

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SUMMARY

Sexual reproduction in plants, unlike that of animals, requires the action of multicellular haploid gametophytes. The male gametophyte (pollen tube) is guided to a female gametophyte through diploid sporophytic cells in the pistil. While interactions between the pollen tube and diploid cells have been described, little is known about the intercellular recognition systems between the pollen tube and the female gametophyte. In particular, the mechanisms that enable only one pollen tube to interact with each female gametophyte, thereby preventing polysperm, are not understood. We isolated female gametophyte mutants named *magatama* (*maa)* from *Arabidopsis thaliana* by screening for siliques containing half the normal number of mature seeds. In *maa1* and *maa3* mutants, in which the development of the female gametophyte was delayed, pollen tube guidance was affected. Pollen tubes were directed to mutant female gametophytes, but they lost their way just before entering the micropyle and elongated in random directions. Moreover, the mutant female gametophytes attracted two pollen tubes at a high frequency. To explain the interaction between gametophytes, we propose a monogamy model in which a female gametophyte emits two attractants and prevents polyspermy. This prevention process by the female gametophyte could increase a plant's inclusive fitness by facilitating the fertilization of sibling female gametophytes. In addition, repulsion between pollen tubes might help prevent polyspermy. The reproductive isolations observed in interspecific crosses in Brassicaceae are also consistent with the monogamy model.

Key words: Pollen tube guidance, Female gametophyte, Prevention of polyspermy, Interspecific cross, *Arabidopsis thaliana*, *magatama*

INTRODUCTION

The exchange of signals between females and males mediates successful fertilization and is one of the central themes in biology. Angiosperms alternate between a diploid sporophytic generation and a haploid gametophytic generation. Gametophytes, which make egg cells and sperm cells, play critical roles in the fertilization. The female gametophyte (FG) of *Arabidopsis thaliana* is seven-celled, composed of an egg cell, a central cell, two synergid cells and three antipodal cells. It develops within an ovule, which is in turn buried in a pistil (Murgia et al., 1993; Christensen et al., 1997; Drews et al., 1998). The pollen grain, which corresponds to the male gametophyte, arises in the anther and is composed of two sperm cells and a vegetative cell. It is delivered to the stigma of the pistil by insects, wind, or direct contact in the case of self-fertile plants like *Arabidopsis*. On the stigma, a pollen tube (PT) germinates from the pollen grain. The sperm cells in the PT are delivered over a long distance to the FG by a process known as pollen tube guidance. In *Arabidopsis*, PTs germinate on the stigmatic papillae cells, penetrate the stigma, and enter into the transmitting tissue. From the bundle of PTs in the transmitting tissue, one PT elongates along the funiculus (the stalk of the ovule), enters the micropyle, and reaches the FG. Two sperm cells are released and double fertilization occurs in the FG (Pruitt and Hülskamp, 1994; Kandasamy et al., 1994; Wilhelmi and Preuss, 1997, 1999; Lush, 1999). It is thought that some mechanism to prevent polyspermy (Wilhelmi and Preuss, 1997) permits only one PT to approach each ovule.

Two models have been proposed to explain the mechanism of PT guidance (Heslop-Harrison and Heslop-Harrison, 1986; Heslop-Harrison, 1987; Pruitt and Hülskamp, 1994; Hülskamp et al., 1995a; Ray et al., 1997), as in the functionally analogous process of axon guidance (Tessier-Lavigne and Goodman, 1996). The mechanical model assumes that PTs elongate along markers present on the surface of the contacting cells. A narrowly defined track could explain why only one PT elongates to each FG. Alternatively, the chemotropic model predicts that PTs grow along a gradient of attracting molecule(s) expressed by target cells or neighbor cells. This model does not readily account for the prevention of polyspermy, so other factors such as repulsion between PTs would be required. In vitro assays of PT guidance suggest the involvement of diffusible molecules such as Ca^{2+}, the transmitting tissue-specific (TTS) protein (Heslop-Harrison, 1987; Cheung et al., 1995), and diffusible molecule(s) from the FGs, as shown for *Torenia* (Higashiyama et al., 1998). However, due to paucity of in vivo data, the two models have long been a subject of speculation and debate (Heslop-Harrison and Heslop-Harrison, 1986; Heslop-Harrison, 1987; Pruitt and

Hülskamp, 1994; Kandasamy et al., 1994; Cheung et al., 1995; Hülskamp et al., 1995a; Ray et al., 1997; Wilhelmi and Preuss, 1997; Higashiyama et al., 1998; Sommer-Knusden et al., 1998; Wilhelmi and Preuss, 1999; Lush, 1999). In the *Arabidopsis pop2 pop3* double mutant, PTs grow in random directions after exiting from the transmitting tissue. Since the phenotype occurred only when both PT and diploid pistil tissues are defective, it is suggested that the POP products function in the adhesion process (Wilhelmi and Preuss, 1996; Smyth, 1997).

The task of the PT guidance system is not only to attract the proper PTs to the FGs, but also to exclude inappropriate pollen. Self pollen is rejected in self-incompatible species, and this is thought to prevent inbreeding depression. (Takasaki et al., 2000). Although incompatibility that acts after the stages of the stigma or style, called late-acting incompatibilities, is not rare, little is know about it (Seavey and Bawa, 1986). Rejection of pollen from other species is known as interspecific incompatibility. It prevents gene flow (reproductive isolation), and thus is thought to the basis of generating new species (speciation). A relationship between self and interspecific incompatibility has been suggested for *Nicotiana*, but little is known about the mechanism of interspecific incompatibility (Murfett et al., 1996).

Theoretical analyses of evolutionary aspects of plant sexual reproduction suggested that gametophytic selection (selection for haploid traits) had contributed to angiosperm evolution (Willson and Burley, 1983; Willson, 1994; Hormaza and Herrero, 1994). In the angiosperm *Hibiscus*, sexual selection for the growth rate of PTs in a pistil was shown experimentally (Snow and Spira, 1991). In some species of ferns, the pheromone antheridiogen emitted by hermaphrodite gametophytes promotes cross fertilization by converting other gametophytes to males (Banks, 1994). However, the paucity of empirical data on the mechanism of reproduction has limited the application of theoretical analyses to other traits such as PT guidance.

Though the PT is guided through diploid pistil tissues, it is known that the FG, the target of the guidance, is also necessary for the guidance to the ovules (Hülskamp et al., 1995a; Ray et al., 1997). When half of the FGs did not develop at all due to a chromosomal rearrangement, no PTs were directed to the FGs, whereas PTs grew normally in transmitting tissue and on ovules with normal FGs (Ray et al., 1997). In *Oenothera*, PT guidance and fertilization might be affected by the genotype of the FG (Heslop-Harrison, 1987). However, very little is known about the interaction between the FG and PT. Here we isolated female gametophyte mutants, and genetically dissected the guidance process.

MATERIALS AND METHODS

Plant material and mapping

The *Arabidopsis thaliana* Wassilewskija ecotype was used. *maa1* and *maa3* mutants were mapped by crossing with the Columbia ecotype, using simple sequence length polymorphisms (Bell and Ecker, 1994). *maa1* showed no recombination in 50 chromosomes with the marker nga280 on chromosome 1. *maa3* is 0.095±0.045 cM from the marker nga8 on chromosome 4.

Microscopy

For scanning electron microscopy (SEM), pistils were fixed and dried following the procedure of Hülskamp et al. (1995a). Alternatively, pistils were dissected, frozen in liquid nitrogen for 1 minute, and observed by SEM. PTs were colored red or green electronically on the resulting images. For Nomarski (differential interference contrast) microscopy, pistils were fixed in a 9:1 mixture of ethanol and acetic acid overnight, having been kept under a gentle vacuum for the first 30 minutes. Then they were treated with 90% and 70% ethanol for 20 minutes each, and cleared in Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, 5 ml glycerol in 30 ml water), and observed by Nomarski optics after dissection. For fluorescence microscopy, pistils were first fixed and cleared for Nomarski microscopy, except that they were not dissected in order to prevent the breakage of PTs. After the phenotypes of each FG had been recorded, the pistils were washed with water and treated with 1 N NaOH for more than 2 hours. The pistils were stained in 0.1% Aniline Blue in 0.1% K_3PO_4 buffer (pH approximately 12.4) for more than 1 hour. Then they were briefly rinsed in the buffer and mounted in glycerol. The samples were observed under UV illumination, to visualize callose of PTs and vascular bundles, and/or by Nomarski optics.

RESULTS

Paths of pollen tubes towards wild-type female gametophytes

After leaving the transmitting tissue, PTs grow on the surface of the funiculus and enter the micropyle (Pruitt and Hülskamp, 1994; Kandasamy et al., 1994; Wilhelmi and Preuss, 1997; Wilhelmi and Preuss, 1999; Lush, 1999). The surface layer of the funiculus is composed of files of cells running proximodistally (Fig. 1A,B, Fig. 4E-H). We examined precisely the paths of PTs using SEM and fluorescence microscopy (Fig. 1A-D). A diagram of ten paths observed by SEM is shown in Fig. 1C, including the paths of the PTs in Fig. 1A,B depicted in red. The following features were apparent. First, no stereotypical path could be found. Second, PTs elongated almost straight along a boundary of cell files of the funiculus in the proximodistal orientation. Even after turning, they elongate straight again along another boundary. Third, as the PTs grew within 10 μm of the micropyle, most (9/10 observations) made an abrupt turn toward the micropyle. In the one exception, the PT elongated on the center line of the funiculus from the beginning and entered the micropyle without turning. In contrast, only four turns were observed in the 50 μm region of the funiculus that is distal to the region described above. This indicates that the paths can be separated into two phases: the funiculus guidance phase and the micropyle guidance phase. The former represents paths on the surface of the funiculus that bring the PT into the vicinity of the micropyle, and the latter indicates paths leading the PTs to the micropyle at the distal funiculus.

Three stages of development of *Arabidopsis* FGs could be defined by anatomical observation. (1) Degeneration of antipodal cells, (2) arrival of a PT to the FG, and (3) degeneration of one synergid cell by penetration of a PT (Schneitz et al., 1995; Christensen et al., 1997; Drews et al., 1998). In contrast, some authors suggest that antipodal cell degeneration occurrs after PT arrival (see Murgia et al. (1993) for discussion). We also found a few FGs with a PT, antipodal nuclei, only one synergid nucleus, two endosperm nuclei and one zygote nucleus (Fig. 1E), confirming that a PT can arrive at an FG before antipodal degeneration. Thus, antipodal degeneration can occur both before and after PT arrival, and

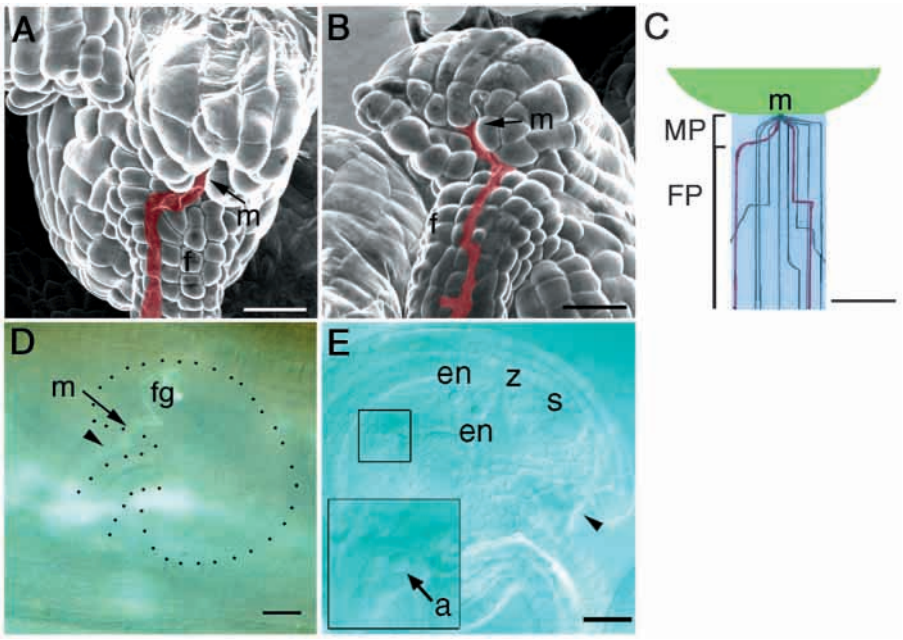

Fig. 1. Wild-type pollen tubes growing toward a wild-type female gametophyte. (A,B) Scanning electron micrographs of pollen tubes (PTs) on a funiculus (f). PTs are colored red. (C) Summary of ten examples of PT paths on the adaxial side of funiculi. The red lines are the paths shown in A and B. PTs elongated almost straight along the funiculus (funiculus guidance phase, FP) then they turned toward the micropyle (micropyle guidance phase, MP). Distal is up. Lines starting at the lateral boundary of the funiculus mean that PTs came from the opposite side of the funiculus. (D) A PT (arrowhead) stained with Aniline Blue elongated on the funiculus, entered the micropyle (m) and reached the FG (fg). The ovule is delineated by the dotted line. (E) A PT arrived before the degeneration of antipodal cells. The other synergid nucleus had already degenerated. a, antipodal nucleus; en, endosperm nucleus; s, synergid nucleus; z, zygote nucleus; arrowhead, PT. Scale bars, 20 μm.

is not necessary for PT guidance or for fertilization (see Discussion).

Isolation of female gametophyte mutants

It is expected that, in a heterozygote with a lethal FG defect, half of the FGs inherit the mutant allele after meiosis and cannot mature into seeds. To isolate FG development mutants, we looked for *Arabidopsis* lines that have only half the normal number of seeds in the siliques (seed pods) (Fig. 2A). We selected 35 lines from 550 families of T-DNA insertional lines. Thirty-one lines showed a similar phenotype: half of the seeds did not develop and half of the pollen grains collapsed in anthers (Fig. 2B). A survey of several lines showed that the FGs did not develop at all and the phenotype could be transmitted both from female and male. We excluded these lines, because it is most likely that the phenotypes were not caused by female gametophytic mutation but by chromosomal rearrangement (Ray et al., 1997; Bonhomme et al., 1998; see Discussion). The other four lines had defects in female gametophyte development. We named the four lines *maa1* to *maa4* (*magatama*, an ancient Japanese comma-shaped bead symbolizing a fetus). In this study, we focused on two mutants, *maa1* and *maa3*, which showed delayed development of FGs.

At the stage when the top of the stamen becomes level with the stigma and pollination occurs, wild-type FGs are at stage FG6 (seven-celled seven-nuclear stage, Fig. 2C, ref. 2). However, FGs of *maa1* or *maa3* mutants remained at stage FG5 (seven-celled eight-nuclear stage), where the two polar nuclei have not fused (Fig. 2D,E). No visible mutant phenotypes were observed in the sporophytic tissues except in the tissues that surround the mutant gametophytes, which would be a secondary effect of the gametophytic defect. Genetic analyses confirmed that both *maa1* and *maa3* were single gametophytic, not sporophytic, mutations (see Table 1). The *maa1* mutation was fully penetrant in FG lethality, and the *maa3* mutation was nearly penetrant. The *maa1* and the *maa3* mutations are not allelic, since they were mapped to chromosome 1 and 4, respectively.

Wandering and polyspermy phenotypes of pollen tubes in *maa1* and *maa3* mutants

Pistils of *maa1* or *maa3* heterozygotes were treated with a

Table 1. Segregation of the *maa1* and *maa3* mutations

	Mutant	Wild type	Segregation ratio (mutant / total)
FGs in *maa1* heterozygote pistils*	182	176	0.508
F_1s of *maa1* (F) × wild type (M)§	0	137	0.000
F_1s of wild type (F) × *maa1* (M)§	25	100	0.200‡
FGs in *maa3* heterozygote pistils*	237	253	0.484
F_1s of *maa3* (F) × wild type (M)§	6	163	0.031
F_1s of wild type (F) × *maa3* (M)§	68	98	0.410‡

The genetics of the gametophytic lethal mutant is reviewed in Drews et al. (1998). In a heterozygote with a female gametophyte (FG) lethal mutation, half of the FGs will show a mutant phenotype. Further, the ratio of mutants in the F_1 progeny of a backcross of a mutant female is expected to be 0. As long as the mutation does not affect male fertility, the ratio of mutant in the F_1 progeny of a backcross of a mutant male is expected to be 0.5. Ratios less than 0.5 occur if the male gametophyte (MG) is partially lethal. The results indicate that the *maa1* mutation displays fully penetrant FG lethality and partial MG lethality, whereas the *maa3* mutation exhibits nearly complete FG lethality and slight MG lethality.

*Numbers of wild-type and mutant FGs were counted in pistils.

§Numbers of wild-type and mutant F_1 progeny plants from the cross between the *maa* heterozygotes and WS ecotype wild type were counted.

‡Significantly smaller than 0.5 ($P<0.01$, χ^2 test).

F, female pistils; M, male pollen grains.

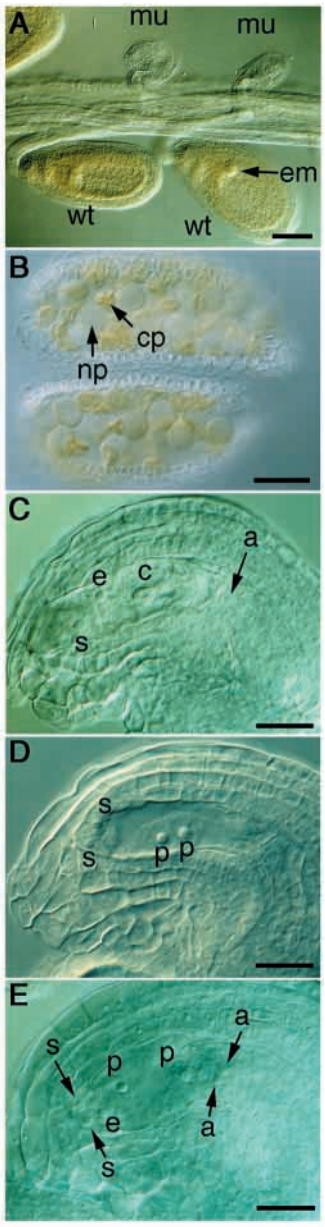

Fig. 2. Female gametophytes of wild-type and *magatama* (*maa*) mutants observed with Nomarski optics. (A) Silique (seed pod) of a *maa1* heterozygote plant. Half of the ovules (wt) are enlarged, and have a developing embryo (em). Comma-shaped ovules (mu) have a mutant female gametophyte (FG). (B) The anther of a line that was discarded in the mutant screening. Half of the pollen grains had collapsed (cp), while the other half were normal (np). (C) Wild-type FG. Central cell nucleus (c), egg nucleus (e), one of the two synergid nuclei (s), and one of the three antipodal nuclei (a) are in focus. (D) FG of the *maa1* mutant. Two polar nuclei (p) of the central cell have not fused and are smaller than those of the wild type. The egg and antipodal nuclei are out of focus. (E) FG of the *maa3* mutant. Two unfused polar nuclei can be seen. Another antipodal nucleus is out of focus. Scale bars, 100 μm (A,B) and 20 μm (C-E).

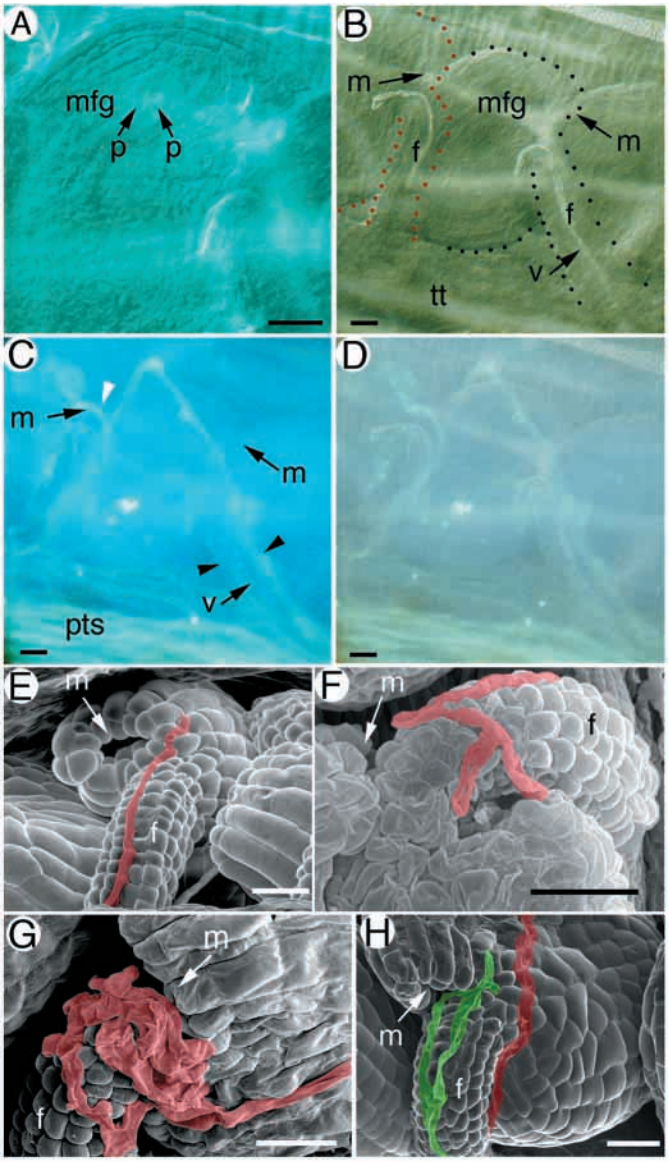

Fig. 3. Aberrant pollen tube guidance in *maa* mutants. (A-D) Two PTs (black arrowheads in C) grew to a *maa3* FG (mfg) but failed to enter the micropyle (m), whereas a PT (white arrowhead) grew to the micropyle of a wild-type FG. Ovules containing a *maa3* FG and wild-type FG are delineated by black and red dotted lines, respectively. (A) Nomarski micrograph of a *maa3* FG at the center (mfg) shows two unfused small polar nuclei. After fluorescence staining with Aniline Blue, the sample was observed with Nomarski optics to show the outline of the ovules (B), with fluorescence to show PTs (C), and simultaneously with fluorescence and Nomarski optics (D). (E-H) Aberrant PT guidance in *maa* mutants. PTs are colored red or green. (E) A *maa3* self-pollinated pistil, (F,H) a *maa1* self-pollinated pistil, and (G) a *maa3* pistil pollinated by WS wild-type pollen grains. In H, two PTs grew on the opposite side of the funiculus (f) and one of them bifurcated, but none entered the micropyle (m). Scale bars, 20 μm.

clearing reagent to identify the mutant and wild-type FGs under a microscope. Then the pollen tubes (PTs) were stained with Aniline Blue. Consistent with the genetic analysis of the progeny plants, FGs showing mutant phenotypes were not fertilized. However, one or two PTs were observed on the funiculus of 32% (n=159) of the *maa1* mutant FGs and 36% (n=132) of *maa3* FGs (Fig. 3A-D; Table 2). The PTs grew to the distal end of the funiculus, but interestingly, failed to grow towards the micropyle. To examine the PT behaviour more precisely, we observed heterozygous pistils by SEM (Fig. 3E-H). We found wandering PTs that were rarely observed in wild-type pistils. The PTs looked as if they had lost their way in the region close to the micropyle and elongated in random directions; some PTs stopped their growth near the micropyle (Fig. 3F), some elongated onto the surface of the integument (Fig. 3C,G), and some turned back to the base of the funiculus. In comparison with the paths of PTs in wild-type pistils, it seems that the funiculus guidance phase was normal, but micropyle guidance phase was absent.

It is noteworthy that two PTs attracted to the same FG was

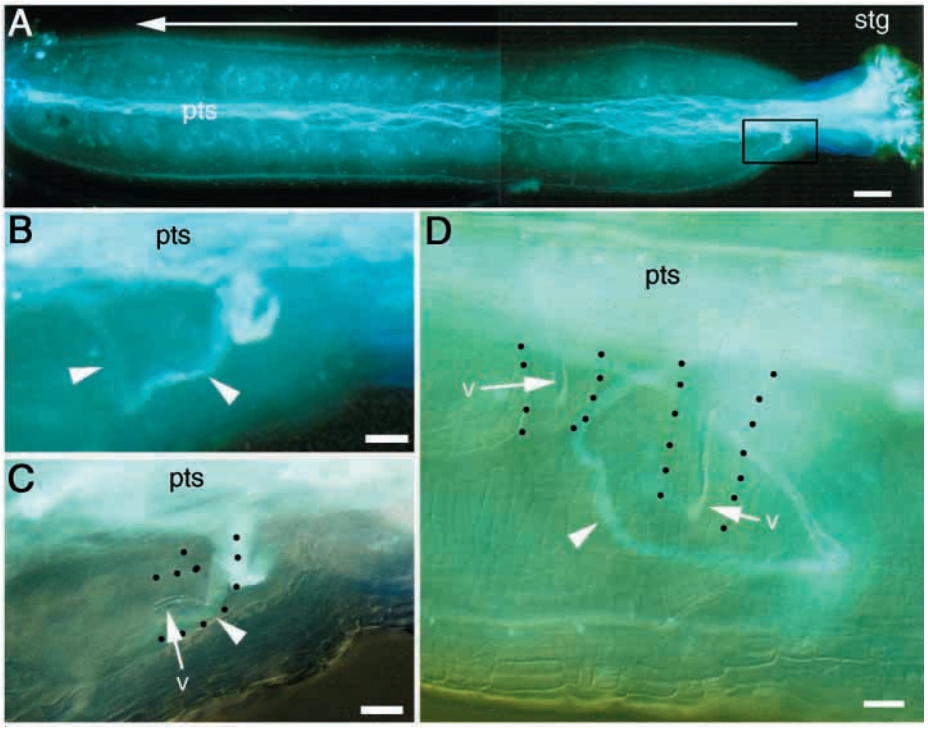

Fig. 4. Paths of PTs in interspecific crosses. (A,B) Fluorescence microscopy, (C,D) simultaneous fluorescence and Nomarski microscopy. *Arabidopsis* pistils were pollinated by pollen grains of *Orychophragmus violaceus* and stained following the procedures described in Table 2. (A) A bundle of PTs (pts) elongated in the transmitting tissue down to the base of the pistil (in the direction of the arrow), but most of the PTs failed to come out of the transmitting tissue; stg, stigma. (B) Enlarged view of the boxed area in A. The PT (arrowhead) elongated on the funiculus but failed to enter the micropyle. (C) Another focus of the same ovule. The funiculus is delineated by dots. v, vascular bundle of the funiculus. (D) A PT came out of the transmitting tissue and wandered independently on funiculi. Scale bars, 100 μm (A) and 20 μm (B-D).

about twice as frequent in mutant as wild-type FGs in the same pistil (Table 2; Fig. 3C,H). This indicates that restriction of the growth of two PTs to an FG is controlled by the FGs and that this restriction contributes to the prevention of polyspermy. Moreover, when two PTs were observed on the same funiculus, the PTs grew parallel at the opposite sides of the funiculus (Fig. 3C,H). This suggests repulsion between PTs.

The wandering phenotype and the polyspermy phenotype are not dependent on defects in PTs, because the same phenotypes were observed when wild-type pollen grains were applied to pistils of *maa* heterozygote plants (Fig. 3G; Table 2). They are not dependent on the defects in sporophytic diploid cells such as funiculus, either, because PTs grew normally towards wild-type FGs in the same pistil of *maa* heterozygotes (Fig. 3C; Table 2). Therefore, the two phenotypes are dependent on the FG, the target of the guidance.

Wandering phenotype in interspecific incompatible crosses in Brassicaceae

We investigated the PT paths in interspecific incompatible crosses using brassicaceous species. When pollen of *Orychophragmus violaceus* germinated on wild-type *Arabidopsis* pistils, the PTs grew into the stigma and elongated into the transmitting tissue. They reached the base of the pistil after 12 hours (Fig. 4A), the same time required for self-pollination. However, the guidance to the ovules was inefficient. Only 3.0% of PTs (13 PTs for 440 FGs) emerged from the transmitting tissue. Of the 13 PTs, six wandered about without elongating on the funiculi (Fig. 4D). Seven showed a wandering phenotype similar to *maa*, elongating along the funiculus but failing to enter the micropyle (Fig. 4B,C). PTs wandered similarly when *Brassica napus* pollen was crossed with wild-type *Arabidopsis* pistils (1.1%, 2 PTs for 187 FGs).

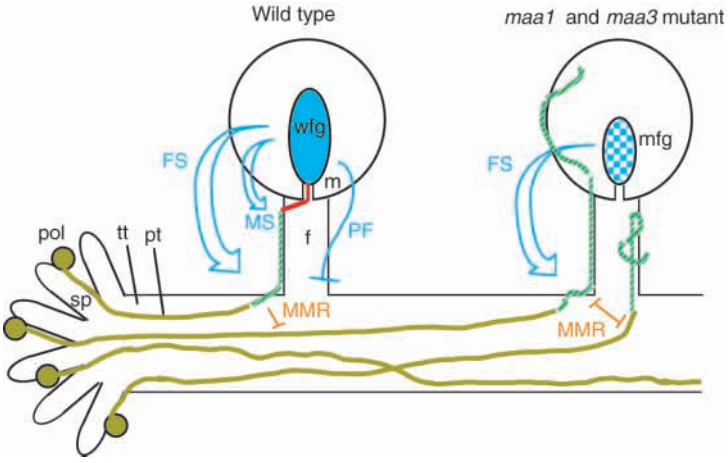

Fig. 5. Schematic drawing of the monogamy model explaining how one pollen tube (PT), but not more, grows to and fertilizes a female gametophyte (FG). The model includes a funiculus guidance signal (FS), a micropyle guidance signal (MS), and prevention of polyspermy by the female gametophyte (PF). The funiculus guidance signal leads a PT to exit the transmitting tissue (tt) and to elongate along the funiculus (funiculus guidance phase, shown by the green path). Then the micropyle guidance signal changes the direction of the PT to the micropyle (micropyle guidance phase, shown by the red path). Male-male repulsion (MMR) may also help prevent polyspermy. The right-hand part of the figure explains our observation that two PTs often grew on the funiculus of the *maa* mutant FG, but failed to reach the micropyle, owing to the loss of MS and PF. f, funiculus; m, micropyle; mfg, mutant female gametophyte; pol, pollen grain; pt, pollen tube; sp, stigmatic papilla; tt, transmitting tissue.

Table 2. Numbers of pollen tubes on the funiculus directed toward the *maa1*, *maa3* and wild-type female gametophytes

	Mutant female gametophyte					Wild-type female gametophyte				
Pistils examined	a	b	b/a (%)	c	c/b (%)	a	b	b/a (%)	c	c/b (%)
Self-pollinated *maa1*	159	51	32.0	20	39.2*	146	128	87.7	21	16.4
maa1 (F) × wild type (M)	146	44	30.1	17	38.6*	121	90	74.4	17	18.9
Self-pollinated *maa3*	132	48	36.4	19	39.6§	120	94	78.3	24	25.5
maa3 (F) × wild type (M)	91	33	36.3	11	33.3§	106	89	84.0	14	15.7
Wild type‡	-	-	-	-	-	178	136	76.4	24	17.6

Pistils were fixed, cleared and examined by Nomarski optics to check the phenotypes of each female gametophyte (FG). Then the pistils were stained with Aniline Blue and the number of the pollen tubes on the funiculus was counted by fluorescence microscopy. We examined pistils of 2.2-2.4 mm length, in which the majority of the wild-type FGs had undergone the first division of the endosperm nucleus. In the case of self-pollinated pistils, this corresponds to the stage when long anthers extend above the stigma. In the cases of crossed pistils, flower buds whose pistil can be seen from the outside were emasculated, pollinated 12 hours later, and fixed after an additional 12 hours. The value of c/b, which represent the ratio of polyspermy, is significantly higher for mutant FGs than for wild-type FGs in each of the four cases (*P<0.01, §P<0.05, χ^2 test).

‡WS ecotype was used. Similar results were observed in Col and L*er* wild-type.a, number of FGs examined; b, number of FGs with one or two PTs; c, number of FGs with two PTs; F, female pistils; M, male pollen grains.

DISCUSSION

Female gametophyte attracts a pollen tube in two steps and excludes other pollen tubes

Based on these results, we propose a model that explains why only one PT grows to one FG in *Arabidopsis*. This monogamy model assumes that an FG provides two different guidance signals, i.e., a funiculus guidance and micropyle guidance signal, and the FG also prevents polyspermy (Fig. 5). The funiculus guidance signal would guide a PT from the transmitting tissue onto the surface of a funiculus. When the PT reaches the distal end of the funiculus, another guidance signal, the micropyle guidance signal, would lead the PT to the micropyle. The FG inhibits other PTs from elongating on the funiculus. This model is supported by the following three experiments. First, investigation of the paths on the funiculus in wild-type pistils showed that it is divided into two phases. In the funiculus guidance phase PTs elongate almost straight. In the micropyle guidance phase they turn abruptly toward the micropyle. Second, *maa*-like wondering of PT on the funiculus in interspecific incompatibility also showed that the paths on funiculus can be divided into two phases. Third, and most importantly, the path of PTs in *maa* mutants provides a genetic dissection of PT guidance by the FG. The funiculus guidance phase is separable from micropyle guidance phase and prevention of polyspermy. These were lacking or had been weakened in the *maa1* and *maa3* FGs, which remained at stage FG5.

Previous studies demonstrated that a PT is not attracted to an ovule when its FG does not develop at all due to a chromosomal translocation (Ray et al., 1997). Therefore, the two guidance signals must be provided at different developmental stages of the FGs; the funiculus guidance signal at or before stage FG5, and the micropyle guidance signal and the inhibiting step after stage FG5. The temporal order of the micropyle guidance signal and the prevention of polyspermy cannot be determined from our data, but it seems reasonable to assume that an FG detects the arrival of a PT and that this detection starts the prevention of polyspermy. In that case, there can be a short time lag before a PT elongating on the funiculus triggers the inhibiting step, which might explain the observation that inhibition of elongation of a second PT on the funiculus is not complete even in wild type.

In the prevention of polyspermy by the FG, an inhibiting signal might work directly on the PT, or indirectly by abolishing or weakening the funiculus guidance signal. Alternatively, it is possible that the FG just stops emitting the funiculus guidance signal upon the arrival of a PT, but this does not seem to be efficient since the guidance molecules would remain for a while.

Morphological observations of FGs have suggested that the synergid cell that is penetrated by a PT has properties of a secretory cell and that the synergid cell might be the source of the diffusible guidance signal (Murgia et al., 1993). In *maa1* and *maa3*, funiculus guidance occurred in the absence of fusion of the two polar nuclei of the central cell. This suggests that maturation of central cell is not necessary for funiculus guidance. In wild type, fertilization occurred in the presence of antipodal cells, in contrast to some previous reports (Schneitz et al., 1995; Christensen et al., 1997; Drews et al., 1998). This suggests that the degeneration of antipodal cells is not necessary for funiculus guidance, micropyle guidance or fertilization. Thus we suggest that egg apparatus, composed of synergid and egg cells, is responsible for funiculus guidance, and possibly also for micropyle guidance. In the in vitro analysis of the PT guidance of *Torenia*, PTs were not attracted to an FG unless both synergid cells were intact (Higashiyama et al., 1998). Our in vivo data supported morphological and in vitro observations that the synergid cell may be responsible for the PT guidance. This conclusion is also consistent with comparative morphological observations that demonstrated antipodal cells persist and the two polar nuclei of the central cell do not fuse at fertilization in maize (Drews et al., 1998).

Gradients of diffusible molecules in pollen tube guidance

Whether chemotropic molecules are involved in PT guidance is a long-standing problem in plant reproductive biology, and the molecular nature of the guidance signals is unknown. We confirmed that there were no stereotypical paths on the funiculus (Hülskamp et al., 1995a). Instead, a PT first elongated along a boundary of funiculus cell files and then turned to the micropyle. This suggests that the PT finds the FG by following a gradient of guiding molecule(s) on the funiculus, rather than by following a predetermined track. Since the FG is genetically necessary for guidance and exerts an attraction over a long distance, the gradient is most likely made by a diffusible chemoattractant, although there remains

a possibility that a gradient of membrane-bound molecules on the funiculus is induced by the FG. In vitro studies using naked FGs of *Torenia* suggest that the PT guidance is mediated by diffusible chemotropic molecule (Higashiyama et al., 1998). In vivo and in vitro data strongly suggest that chemotropic diffusible factors guide PTs near FGs.

If there is a concentration gradient, is there any reason why two distinct guidance signals would be needed? A mathematical model of axon guidance (Goodhill, 1998; Lush, 1999) showed that there exists a maximal distance over which one attracting signal can make a functional gradient. If the substance is produced at higher levels, it can influence a wider region, but cannot act as a directional signal near the source because postulated receptors would be saturated. In the case of PT guidance, the funiculus guidance signal would work over a long distance from the transmitting tissue to the distal end of the funiculus, whereas the micropyle guidance signal would be required for guidance over a relatively short distance.

The chemotropic molecules and the adhesion molecules are not mutually exclusive. Adhesion may be a prerequisite for a chemotropic molecule to work. That the PT elongated along the boundary of funiculus cell files might reflect some favorable surface property, or, might reflect the distribution of guidance molecules. In addition, it is plausible that PTs are guided mechanically in other steps such as elongation in the transmitting tissue (Heslop-Harrison, 1987; Smyth, 1997; Lush, 1999).

In gymnosperms, such as conifers and gnetophytes, pollen grains are deposited close to the micropyle and grow towards FGs. Morphological and in vitro data suggest a diffusible guidance factor in the conifer, *Pseudotsuga menziesii* (Fernando et al., 1997; Takaso and Owens, 1997). One possibility is that the PT guidance of gymnosperms and micropyle guidance of angiosperms are homologous systems and that angiosperms have evolved an additional guidance mechanism when their seeds (=sperm) were enclosed (=angio) as its name indicates.

Prevention of polyspermy and inclusive fitness

Angiosperm sexual reproduction involves five tissues with distinct genomic compositions: diploid pistil tissues, haploid female gametophyte, haploid pollen tube, diploid embryo, and triploid endosperm. The strategy employed by each part may result in competition or cooperation. Analysis of the *MEDEA* gene supports the intragenomic conflict theory (Grossniklaus et al., 1998; Kinoshita et al., 1999). The presence of the *medea* mutation in an FG resulted in the overproduction of endosperm, and a *MEDEA* gene inherited from the male parent was not transcribed in the endosperm due to imprinting. Since multiple males may successfully fertilize one female, there exists a competition between genetically different embryos for nutrients. As a result, the paternal component would 'strive' to extract nutrients for their own offspring, and *MEDEA* is a tool for the mother to inhibit that effect. Competition also plays a key role in regulation of PT growth rates (Willson and Burley, 1983; Snow and Spira, 1991; Willson, 1994; Hormaza and Herrero, 1994).

Prevention of polyspermy is critical for successful sexual reproduction because fitness would be severely reduced if all the PTs grew to the nearest FG. As described above, a mechanism directed by the FG prevents polyspermy. Sibling FGs share half of their genes. The prevention of polyspermy by FGs can facilitate the fertilization of sibling FGs by providing more PTs. Thus it can increase inclusive fitness, similar to the altruistic behavior of haploid female worker bees that help their close relatives. This shows a contrast to the competition after fertilization between embryos, which share fewer genes due to the contribution of male genome. This idea could be tested by identifying mutants defective in the prevention of polyspermy and measuring their fitness.

This might not be the sole mechanism for prevention of polyspermy, because not all *maa1* and *maa3* FGs attracted two PTs and because not more than two PTs elongated on one funiculus. The observation that two PTs grew at the opposite sides of the funiculus may indicate a repulsion process between PTs. This repulsion could also help the prevention of polyspermy (Fig. 5). It may result from male-male competition by means of a diffusible repellent, as suggested in competition among pollen grains on the stigma (Hormaza and Herrero, 1994), and the prevention of polyspermy would be a by-product of the competition. Alternatively, a female-mediated mechanism, such as consumption of nutrients, might result in other PTs avoiding pre-occupied FGs.

Interspecific incompatibility and reproductive isolation

When pollen grains of brassicaceous species were crossed to *Arabidopsis* pistils, most of them penetrated the stigma (Hiscock and Dickinson, 1993; Kandasamy et al., 1994; Hülskamp et al., 1995b). At later stages in the crosses with *Orychophragmus* and with *Brassica*, we found that the crosses are incompatible because the PTs failed to arrive at the FG. The observation that only a fraction of the PT emerged from the transmitting tissue indicates that the funiculus guidance signal was absent or ineffective. After exiting the transmitting tissue, about half of the PTs showed *maa*-like wandering on the funiculus. This suggests that the micropyle guidance signal did not attract PTs of the different species. We observed similar pattern in some other brassicaceous species tested (K. K. S. and K. O., unpublished data).

The wandering phenotypes present a marked contrast to the growth arrest of self-incompatible PTs in pistils of Brassicaceae and Solanaceae (de Nettancourt, 1977; Seavey and Bawa, 1986; Hiscock and Dickinson, 1993; Murfett et al., 1994; Takasaki et al., 2000). We suggest that interspecific incompatibility in *Arabidopsis* pistils can be attributed to the loss of proper PT guidance by diversification of the guiding molecules and/or their receptors. Selecting PTs of the same species accurately from diverse PTs might be a low-cost strategy for interspecific incompatibility, rather than inhibiting the growth of incompatible PTs.

Isolation of female gametophyte mutants

Several groups reported the screening of female gametophyte mutants (reviewed by Drews et al., 1998). One strategy is to find segregation distortion of an antibiotic resistant gene on T-DNA inserted in the plant genome. However, Bonhomme et al. (1998) reported that disruption of gametophytic genes is unexpectedly rare and cannot be inferred from segregation distortion alone. They suggested that chromosomal rearrangement may have caused the segregation distortion. In our screening, as much as 6% (31/550) of T-DNA insertional

lines seemed to have chromosomal rearrangements, in which half of the seeds did not develop and half of the pollen grains collapsed in the anthers (Ray et al., 1997; Bonhomme et al., 1998). So in our screening for female gametophyte mutants, we excluded lines in which half the pollen grains collapsed. We suggest that this is an efficient strategy for isolating female gametophyte mutants, though mutants that have defects in early pollen development may be dismissed.

The molecular nature of the signaling mechanism proposed in our model should be clarified. Because biochemical approaches to isolate the signaling molecules have not been successful so far, it will be necessary to take genetic strategies using a variety of *Arabidopsis* mutants. Factors in the guidance mechanism will be found as gametophytic mutants predicted in our monogamy model.

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